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Clinical grade adult stem cell banking

Sreedhar Thirumala,[1] W. Scott Goebel[1,2] and Erik J. Woods[1,3,*]

[1]General Biotechnology LLC; [2]Department of Pediatrics (Hematology/Oncology) and Wells Center for Pediatric Research; [3]Department of Microbiology and Immunolgy; Indiana University School of Medicine; Indianapolis, IN USA;

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There has been a great deal of scientific interest recently generated by the potential therapeutic applications of adult stem cells in human care but there are several challenges regarding quality and safety in clinical applications and a number of these challenges relate to the processing and banking of these cells ex-vivo. As the number of clinical trials and the variety of adult cells used in regenerative therapy increases, safety remains a primary concern. This has inspired many nations to formulate guidelines and standards for the quality of stem cell collection, processing, testing, banking, packaging and distribution. Clinically applicable cryopreservation and banking of adult stem cells offers unique opportunities to advance the potential uses and widespread implementation of these cells in clinical applications. Most current cryopreservation protocols include animal serum proteins and potentially toxic cryoprotectant additives (CPAs) that prevent direct use of these cells in human therapeutic applications. Long term cryopreservation of adult stem cells under good manufacturing conditions using animal product free solutions is critical to the widespread clinical implementation of ex-vivo adult stem cell therapies. Furthermore, to avoid any potential cryoprotectant related complications, reduced CPA concentrations and efficient post-thaw washing to remove CPA are also desirable. The present review focuses on the current strategies and important aspects of adult stem cell banking for clinical applications. These include current good manufacturing practices (cGMPs), animal protein free freezing solutions, cryoprotectants, freezing & thawing protocols, viability assays, packaging and distribution. The importance and benefits of banking clinical grade adult stem cells are also discussed.

Introduction

Adult stem cells offer great therapeutic promise for a diverse range of medical applications. They can be derived from different tissues of the body including bone marrow, blood, fat, dental pulp, placenta, liver and brain.[1-3] To date, the hematopoietic stem cells (HSCs) originating in bone marrow have arguably been the most extensively studied and are the first adult stem cells to be used successfully in therapy.[4,5] Besides HSC, bone marrow also contains a population of stromal cells that can differentiate into non-hematopoietic lineage. Commonly termed as mesenchymal stem cells (MSCs), these cells have shown to possess multipotentiality when induced ex vivo. While MSC are traditionally isolated from bone marrow, over the last few years, various other sources have been lucratively explored for the presence of mesenchymal like stem cells. These include but are not limited to; adipose tissue, liver, brain, cord blood, placenta and dental pulp.[6-12] When isolated by plastic adherence and expanded ex vivo, these cells show a broad spectrum of differentiation potential from cell types of mesodermal origin, like osteoblasts, adipocytes, chondrocytes to ectodermal (neuronal) and endodermal (hepatocytes) origins (**Figure 1 and 2**).[12,13]

While MSCs derived from diverse tissues share some common stem cell properties, they significantly differ in terms of their population numbers in host tissues and their ability to proliferate and differentiate ex vivo. For example, MSCs are a rare population in bone marrow constituting only 0.002% of total stromal cell population.[14,15] Whereas adipose tissue harbors nearly 2% MSCs in its stromal vascular fraction.[14,15] As compared to bone marrow, further reduced frequency of MSCs is reported for cord blood stem cells.[16,17] Gronthos et al.[10] reported that colonies of dental pulp cells occur at an apparently higher frequency in comparison to bone marrow over similar plating densities. Furthermore, comparative analysis of MSCs derived from various tissues suggest that although they fulfill the general accepted criteria for MSCs, they markedly differ in terms of growth rates, differentiation abilities, colony frequencies, expansion rates, success rates of isolation and gene expression profiles beyond donor and experimental conditions.[17-23]

Perhaps the most attractive aspect of these MSCs is that they can be maintained and propagated in culture for long periods of time without losing their differentiation capacity generating large cell quantities appropriate for clinical applications. Although bone marrow has been the most common source for MSCs, the significant drawback is that the number of bone marrow MSCs and their differentiation potential significantly decrease with age.[20,21,24] The isolation of MSCs from bone marrow is highly invasive and causes pain and discomfort to the patient. In addition to that, the frequency of bone marrow MSCs also depends on the volume of aspiration. The concentration of MSCs per milliliter decreases with increased volume of aspirated marrow for each puncture because of dilution of the bone marrow sample with peripheral blood.[25] These major drawbacks inspired many investigators to explore alternative tissues for more abundant and accessible sources of MSCs with least invasive collection procedures.

*Correspondence to: Erik J. Woods; Email: erik@gnrlbiotech.com
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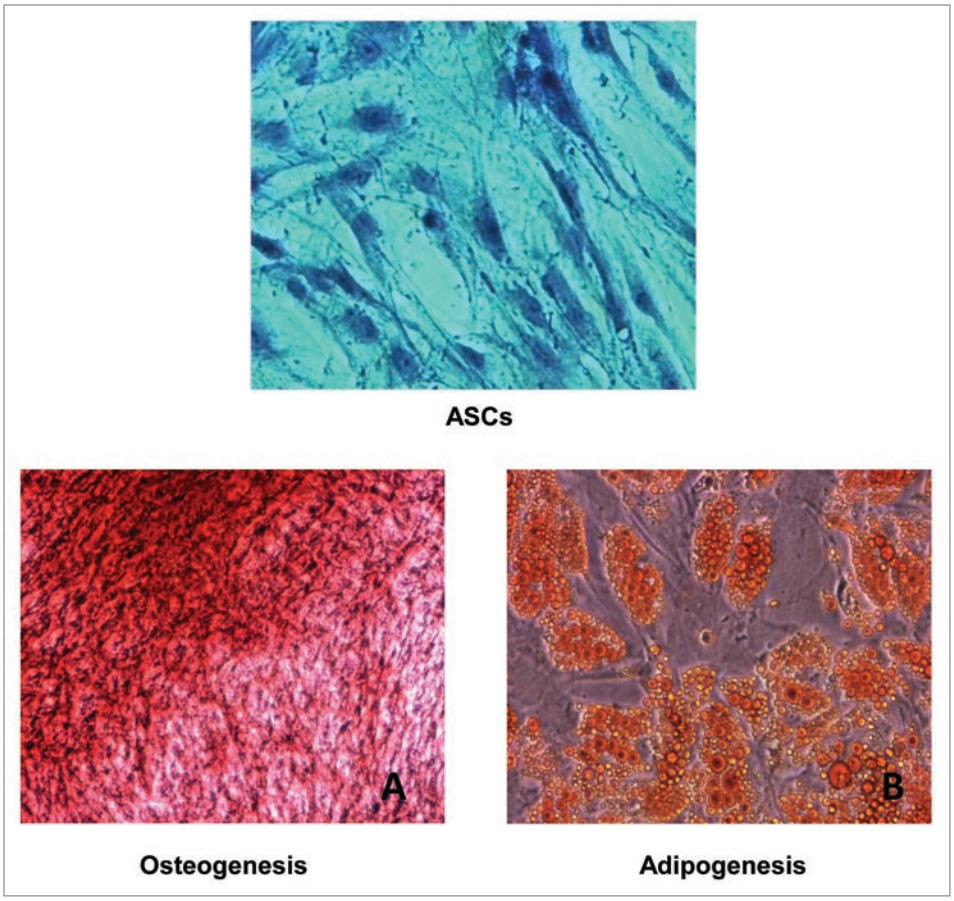

Figure 1. In vitro differentiation potential of frozen-thawed adipose derived adult stem cells (ASCs). When culture expanded, ASCs exhibit a spindle shaped fibroblastic morphology (Top image, stained with Toludine blue, 40X). Under appropriate inducing conditions, the culture of frozen thawed ASCs will demonstrate adipogenic differentiation as evidenced by lipid accumulation (A, stained with Oil Red O, 40X) or osteogenesis as seen by calcium deposition (B, stained with Alizarin Red, 10X).

Various preclinical and clinical studies present dramatic examples that illustrate the therapeutic value of MSCs. These cells hold great promise in human therapy not only due to their multipotentiality and expansion rates, but also due to their immunosuppressive properties.[13,26,27] This advantage of MSCs over other cell types allows allogeneic transplantation of these cells without the need for immunosuppression. This has important implications for the therapeutic application of MSCs because MSCs derived from healthy unmatched donor can be cryopreserved, thus making them available in a timely manner for patients in a variety of acute and chronic clinical settings.[13] Moreover, MSCs are robust and can survive freezing temperatures without any significant loss in viability, thus allowing for future "off-the-shelf" therapy approaches.[5,28,29]

In a clinical transplant or transfusion application, a large number of frozen stored MSCs are usually required and therefore the development of stem cell banks is necessary. These banks must assure the quality and safety of these cell products especially when the stored MSCs are intended for clinical use in cell therapy and regenerative medicine. Regardless of the success achieved in cryopreservation storage of MSCs at laboratory scale, many important technical and medical issues remain with respect to the production and storage of these cells for clinical applications. One pragmatic issue is the development of large-scale manufacturing methods with appropriate quality assurance and quality control to generate cells in compliance with current Good Manufacturing Practices (cGMP).[30] In general, principles of cGMP have to be applied for the entire process from cell collection to freezing and storage of the cells.[31,32] This has to be achieved through the development and introduction of validated SOPs under cGMP guidelines with appropriate quality assurance programs that comprise microbiological, environmental, biosafety and quality assurance methods. The other significant concern is the development and usage of cGMP grade reagents free of animal serum protein in cell isolation, processing and storage. Published evidence shows that serum proteins used in the cryopreservation media is difficult to remove during washing and any residue left can trigger adverse reactions in patients who receive cell infusions or transplants. Therefore, development of serum free media for the processing and storage of MSCs meant for clinical use is a critical issue. In addition, it will be valuable to develop non-toxic cryoprotectants (CPAs) for the cryopreservation and storage of MSCs. Many CPAs, such as dimethyl sulfoxide (DMSO), are potentially toxic at the molar concentrations they are often used to preserve cells, which complicates direct use of frozen thawed cells in patients. Furthermore, addition and removal of these CPAs are complex processes associated with detrimental osmotic shock to the cells.[5] Therefore, the development of CPA free media or non-toxic CPAs are certainly required in order to enhance the usefulness of MSCs for clinical applications. Finally, the development and validation of closed containers for storing, packaging and distribution of cells for clinical use that minimizes the risk of cross contamination between the samples while protecting the personnel from the potential risk of blood borne pathogens will be needed.[30] The ideal container systems should be suitable to package, to store and to transport cell therapy products at lower temperatures, and meet pharmaceutical quality requirements to maintain cell viability over its intended shelf-life. In this review we attempt to outline some of the current technology in the cryopreservation and banking of clinical grade adult stem cells for therapeutic applications. Due to the inherent breadth of the topic, we restrict our discussion to clinical banking of mesenchymal stem cells (MSCs)

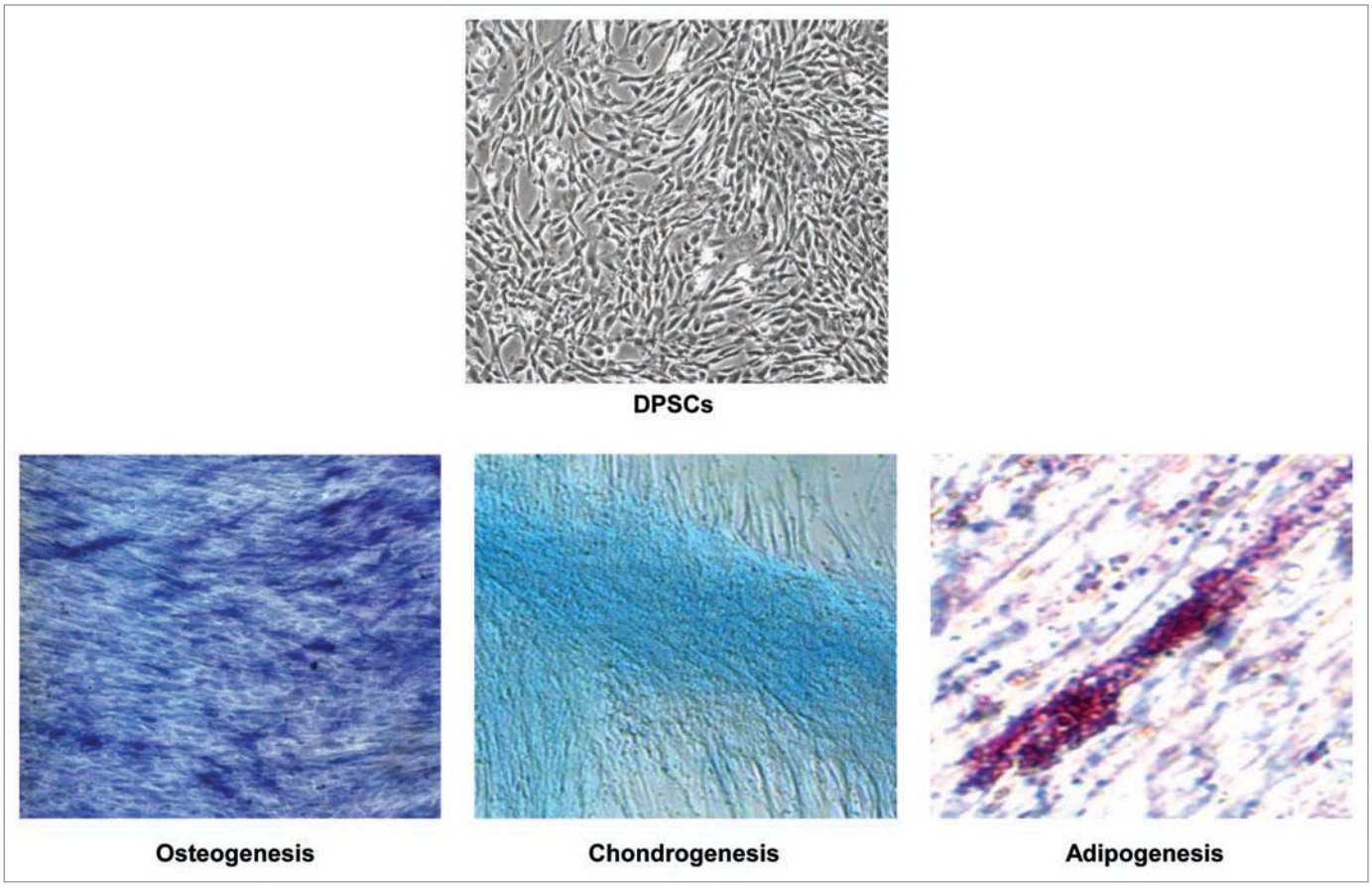

Figure 2. Dental pulp stem cells (DPSCs) recovered from frozen thawed pulp tissue expanded to passage 3 were then cultured in osteogenic (A), chondrogenic (B) or adipogenic (C) differentiation medium for three weeks before staining with an alkaline phosphatase kit, Alcian blue, and Oil red O respectively.

derived from fat, dental pulp, placenta and amniotic fluid as these tissues have been shown to be the most accessible and abundant sources for MSCs. Published research shows that these tissues are rich in MSCs and can potentially act as dependable reserves for the production and storage of MSCs in large quantities required for clinical transplantation and transfusion application.

Clinical Banking of Adipose Derived Adult Stem Cells (ASCs)

Human adipose tissue provides a uniquely abundant and accessible source of adult stem cells.[6,30] Successful cryopreservation and banking of scientifically and commercially important adipose derived stromal/stem cells (ASCs) under cGMP conditions would revolutionize tissue engineering and regenerative medicine industry.[30,33,34] To date, cGMP culture and storage techniques for ASCs have not been published, and the existing methods are fairly unsophisticated and do not meet the quality criteria set by the FDA.[35] Therefore it is of paramount importance to devise and develop relatively simple, safe and effective methods to process and store ASCs before widespread use in humans. As we discussed earlier, for providing a reasonable assurance of the absence of contamination and for providing a reliable source of cells for human clinical applications, the principles of cGMP have to be imposed for the entire process from ASCs collection to freezing and storage of the cells.

At present clinically relevant survival rates for adipose derived adult stem cells (ASCs) are only achieved through the use of cryopreservation solution containing animal serum protein plus cryoprotective agents (CPAs) or growth medium containing serum plus CPA.[28,33,34,36] Addition of cryoprotectants is a common practice during cryopreservation to reduce the freezing induced damage to the cells. There is a large divergence in choice of CPAs, ranging from low molecular weight solutes (permeating) like dimethyl sulfoxide (DMSO) and glycerol, to sugars like sucrose, and trehalose, to high molecular weight polymers (non-permeating) like polyvinylpyrollidone (PVP) and hydroxyl-ethyl-starch (HES).[37] Currently, most adipose derived stem cell (ASC) cryopreservation protocols use the plasma membrane-permeating molecule dimethylsulfoxide (DMSO) as CPA.[28,33,34,36,38,39] DMSO is also a common CPA of choice for other adult stem cells including stem cell derived from cord blood, dental pulp and placenta.[5,40,41] This is arguably a good choice because DMSO is available in clinical grade preparations and is known to be much more successful than other CPAs for most cell types, often being a 'default' cryoprotectant for research laboratory use as well.

While virtually all clinical banks use DMSO in their cryopreservation processing, and to date the most appropriate concentration for ASCs is 10% v/v. Liu et al.[36] demonstrated that cryopreservation of ASCs using 10% DMSO as CPA has no effect on the phenotype, proliferation or osteogenic differentiation. Goh et al.[34] recently reported optimal cryopreservation results using 10% DMSO as evaluated by membrane integrity and CFU (colony forming unit) analysis. They further demonstrated that the post-thaw viability of ASCs is a function of storage concentration and that an optimal viability was observed for a concentration of 0.5 x 10^6 cells/ml. In addition to that, previous studies analyzed the fundamental cryobiological characteristics of ASCs using calorimetry and a mathematical model to determine theoretically optimized procedures for cryopreserving these cells in the presence of 10% DMSO and 10% Glycerol as CPAs.[33,39] These studies indicated statistically significant change in the freezing response when either DMSO or glycerol is used as CPA. In a recent study, Fuller and Devireddy[38] examined the effect of two different freezing methods (controlled rate freezing and directional freezing) on the membrane integrity of ASCs when 10% DMSO is used as CPA. Under comparable cooling conditions, they observed the post-thaw viability of cells frozen using the directional freezing were significantly lower than those obtained using controlled freezing. These observations hold up the argument that apart from the imposed thermal conditions during freezing, the choice of CPA, the storage concentration and the method of freezing also play a vital role in the optimal cryopreservation of ASCs.

Although DMSO is regarded as relatively non-toxic, the clinical use of frozen-thawed cells treated with DMSO can cause many adverse effects and toxic reactions.[42-49] It has also been reported that DMSO is not only cytotoxic but it also induces differentiation of stem cells to cardiac or neuronal like cells when added to the cell culture medium.[50,51] Therefore, it is of vital necessity to reduce the toxicity by the removal of DMSO prior to clinical use. However, the total removal of DMSO from the frozen-thawed cells is complex and time consuming.[42,43] Therefore, it is essential to develop cryopreservation protocols either with lower concentrations of DMSO or with non-toxic alternatives to DMSO. A review of the published literature over the past several years identifies several reports suggesting that adult stem cells (hematopoietic and non-hematopoietic) can be successfully cryopreserved with minimal concentrations of DMSO.[45,52-62] For example, Zhao et al.[57] showed that the use of 5 or 10% DMSO produced similar results during cryopreservation of fetal human liver $CD34^+$ cells. Sputtek et al.[60] reported optimal cryopreservation of peripheral blood HPCs using at least 5% DMSO. Abrahamsen et al.[61] showed that cryopreserving peripheral blood progenitor cells (PBPC) with 5% DMSO instead of 10% DMSO produced improved ASC viability while Stiff et al.[62] developed an alternative cryopreservation protocols for hematopoietic stem cells using 5% DMSO in conjunction with 6% hydroxyethyl starch (HES). Furthermore, Liseth et al.[45] concluded that that the post-thaw survival for $CD34^+$ was almost identical when frozen with either 4 or 5% DMSO but further lowering the concentration of DMSO to 2% had an adverse effect on post-thaw cell survival. Yet, to this date no study has investigated or reported the effect of reduced concentrations of DMSO during cryopreservation of ASCs. Our recent studies indicated intriguing observations when investigated the minimal concentrations of DMSO during cryopreservation of ASCs. We observed no significant differences in ASC cell viability and apoptosis when cells were cryopreserved with DMSO concentration ranging between 2 and 10%. However, a further decrease in DMSO from 2 to 0% was extremely detrimental. This critical finding suggested that there must be a "minimal" threshold concentration of DMSO between 0 and 2% that should successfully cryopreserve ASCs without any significant loss in viability (unpublished data).

Another major constituent of the media used for the cryopreservation of ASCs is serum derived from animals. It is common practice to include serum in solutions used for frozen stocks as a source of nutrients and other perceived benefits during post-thaw cell culture. However, the animal serum contains undesired source of xenogenic antigens and bears the risk of transmitting animal viral, prion and zoonose contaminations.[63,64] Hence, the proposed criteria for cGMP require that animal-derived supplements should, whenever possible, be removed or replaced with synthetic recombinant supplements. In contrast to animal serum, the use of autologous serum is advantageous because it eliminates the risk of infectious disease transmission completely. A closer look into the literature shows that, a multitude of work has been carried out to show that autologous serum is nearly identical from animal serum with regard to isolation, expansion and differentiation.[65-69] For example, Matsuo et al.[68] reported that autologous serum provided sufficient ex vivo post-thaw expansion and osteogenic differentiation of patient own MSCs cryopreserved in a serum free solution. In another study, cryopreservation of peripheral blood stem cells (PBSC) in autologous serum did not jeopardize their engraftment potential.[69] A drawback, however, is that the production of autologous serum is a costly process and requires a preoperative blood donation by the patient.[70] This negative aspect restricts the use of autologous serum for small clinical studies and will not be appropriate for future off-the shelf availability of stem cell therapy.[71] Additionally, to overcome the disadvantages associated with animal or autologous serum, it is also possible to use allogeneic human serum in cryopreservation. However, the use of allogeneic human serum may not completely eliminate the risk of infectious disease transmission[70] and hence efforts need to be made to reduce, and preferably remove serum (animal and human) in freezing (cryopreservation) media. But, to date, not much attention has been paid to the development of serum free cryopreservation media for MSCs. This may, in part, due to the fact that the frequency of MSCs in many tissues is relatively low and the cells stored in serum free conditions are eventually required to be expanded ex vivo to transplantable numbers either in serum containing or serum free culture media. Current research shows that many studies already investigated the serum free expansion of MSCs ex vivo and produced contradictory results.[72-77] However, since adipose tissue can yield huge amounts of stem cells and can be obtained in abundance,[14,30] it is realistically feasible to store large amounts of autologous ASCs derived from the patient's own fat in serum free conditions, so

that for any potential future use the cells can be directly transferred to clinics without any further expansion ex vivo. The enormous potential of ASCs in clinical therapy is clear and we believe the time is ripe for the development of serum free cryopreservation methods for clinical banking of ASCs. In a recent review, Gimble et al.[30] anticipated that future citizen may donate their fat in "fat drives" to donate their fat similar to the individuals around the world participate in "blood drives" to donate their blood for medical treatment.

ASCs are routinely cryopreserved and stored in single cell suspension. Given the potential contaminations associated with extensive up-front processing, a simpler choice would be the cryopreservation storage of stromal vascular fraction of adipose tissue for direct post-thaw use in clinical therapy. However, it is very important to evaluate the impact of other cellular components that are present in the stromal vascular fraction when this cell population is directly used in therapy.[78] A more ideal choice would be the cryopreservation of adipose tissue as a whole, with the idea that stem cells could be extracted post-thaw. The rationale for this would be to preserve clinical samples for subsequent stem cell recovery as it is reasonable to speculate that cryopreservation of tissues in the clinic will be more practical than direct primary isolation of stem cells, which would require additional equipment and personnel. In a recent study, cryopreservation of finely minced cord blood tissue in autologous cord plasma produced promising results when the thawed tissue section were cultured ex vivo for the growth and expansion of MSCs.[79] In fact, cryopreservation of fat tissue is not a new research area and has been extensively investigated in large scale adipose tissue engineering, especially in applications of maxillofacial and craniofacial surgery. Although many studies found that freezing fat grafts by using optimal cryopreservation techniques enables their long-term preservation,[80-86] only few considered whether the frozen fat tissue could still be a reliable source of adult stem cells.[87,88] Further studies are required to determine whether cryopreserved fat tissue still maintains the viable and potent adult stem cells in quantities required for clinical needs.

Clinical Banking of Dental Pulp Derived Stem Cells (DPSC)

Stem cells derived from dental pulp are viewed as having promising clinical potential and could provide an alternative source of MSCs for human therapy. Indeed, stem cells obtained from dental pulp of third molar (wisdom teeth), periodontal ligament and periapical follicles of adult teeth as well as from exfoliated/extracted deciduous teeth were identified by their ability to form clonogenic cell clusters and to differentiate into various other cell types (**Figure 2**).[29,89,90] The immunomodulatory properties of these cells make them suitable as candidates for new allogeneic stem cell based therapies.[91] The extracted human wisdom teeth represent an easily accessible, often discarded tissue that may be a valuable source of MSCs for future research and clinical applications. In a series of recent studies, our group and others have demonstrated that post-thaw stem cells derived from dental pulp (often referred as dental pulp stem cells, DPSCs) retain their viability and ability to differentiate after cryopreservation storage signifying that it is possible to freeze and bank DPSCs for future therapeutic applications. Zhang et al.[92] reported no loss in differentiation ability of frozen stored pulp derived stem cells from third molars when compared with non frozen controls. After two years of cryopreservation storage in liquid nitrogen, dental pulp stem cells were able to differentiate and proliferate normally.[93]

A major limitation in tissue banking is the degradation of the tissue of interest between the time of harvest and cryopreservation.[94] Banking of DPSCs would be greatly aided if DPSCs isolation could be delayed for several days to allow for transport of teeth to the tissue bank for processing. To this end, our group recently reported a study in which the processing of dental pulp-derived MSCs (DPSCs) from harvest to storage was considered using large numbers of teeth.[41] In that study we reported that isolation of DPSCs is feasible for at least 120 h after tooth extraction and that cryopreservation of established early passage cultured DPSCs leads to high-efficiency recovery after thawing. In a more recent study,[29] we further established that DPSCs can be stored at -85°C or -196°C for at least six months without loss of functionality. It was also determined that DPSCs viability after cryopreservation is not limited by the concentration of cells frozen, at least up to 2 x 10^6 cells/mL. These promising cryobiological properties as well as their multi-potentiality make DPSCs an ideal choice for clinical banking under cGMP so that the autologous pulp stem cells can be stored until they may be needed later in life.

Most of the published cryopreservation protocols for DPSCs employed freezing media containing 10% DMSO in fetal calf serum (FCS).[29,41,93] There are currently no published data on cryobiological characteristics of DPSCs at reduced concentrations of DMSO. However, in our recent effort to devise clinical grade cryopreservation protocols, we have evaluated post-thaw viability of DPSCs at reduced DMSO concentrations. These experiments revealed that reducing DMSO from 10.5 to 7% did not show any noticeable change; however further reduction in DMSO concentration demonstrated significant loss in viability.[29] Nevertheless, these findings suggest that cryopreservation of DPSCs at lower DMSO concentrations is indeed feasible. Further cryopreservation studies by partially or completely replacing DMSO with non-penetrating high molecular weight polymers (e.g., polyvinylpyrrolidone or polyethylene glycol or trehalose) are obviously required. For cGMP production, the elimination of serum products is also another prerequisite. However, to the best of our knowledge, no study has yet investigated the serum free cryopreservation of isolated DPSCs; although several groups published ex vivo expansion of DPSCs either in reduced serum or serum deprived conditions.[95,96]

Isolating DPSCs can be laborious, time-consuming and expensive, especially while employing current good manufacturing practice (cGMP) compliance for clinical use of the cells; therefore, cryopreservation of whole teeth or isolated tooth tissues may be advantageous for the banking of specimens from which DPSCs cultures are not immediately needed. Our previous preliminary experiments have demonstrated recovery of viable DPSCs after cryopreservation of intact teeth, suggesting that minimal processing may be adequate for the banking

of samples with no immediate plans for expansion and use.[41] However, our more recent experiments indicated that expansion of DPSCs from cryopreserved intact teeth is not reliable or repeatable.[29] On the other hand, the optimized results with the least manipulation were achieved by isolating and cryopreserving the tooth pulp tissues, with digestion and culture performed post-thaw. These results were similar to the published data on the DPSCs isolation from cryopreserved pulp tissue,[41,92,97] although at least one other group[93] obtained completely negative results cryopreserving whole pulp tissues. Further, contemplating clinical use of DPSCs, we compared the results of the pulp tissue cryopreservation experiments using medium with fetal calf serum to a commercially available defined medium (Cryostor-CS-10) specifically indicated for clinical banking, which worked equally well.[29] These results imply that minimal tooth processing may be ideal for the banking of samples with no immediate plans for DPSCs expansion and use, which in turn may limit costs and facilitate clinical banking of this potentially important cell type. Furthermore, it was also apparent that minced pulp tissue can be cryopreserved in a defined serum free freezing media.

Clinical Banking of Placenta and Amniotic Fluid Derived Stem Cells

Placenta and amniotic fluid (AF) have recently emerged as other potential sources of well characterized mesenchymal stem cells (MSCs). The MSCs derived from these sources have been shown to differentiate into functional cells corresponding to each of the three main embryonic germ layers (ectoderm, endoderm and mesoderm) and given rise to several other body cell types.[98] With millions of live births every year, placenta and AF, typically considered medical waste, could feasibly provide more abundant sources for mesenchymal stem cells (MSCs) than any other part of the human body. Therefore, sourcing MSCs from placenta and AF could be relatively easy and long term banking of placenta derived mesenchymal stem cells (PDSCs) and AF derived stem cells (AF-SCs) will potentially have a greater impact on future regenerative medicine.

Unlike other tissue sources, placenta has complex structure created from several maternal and fetal tissues. At present, many researchers have successfully isolated mesenchymal like stem cells from various components of placenta, including: whole placenta,[99-102] the amniotic membrane,[9,103,104] the chorionic membrane,[104,105] the internal area of placental lobules,[106] the deciduas membranes,[9,107] and chorionic villi.[108,109] Amniotic fluid that is enclosed by amniotic membrane of the placenta to protect and cushion the fetus is also shown to be a rich source of MSCs.[110,111] While MSCs derived from amniotic fluid are fetal in nature, differing results have been published regarding the source of the MSCs present in placental tissues. For example, in't Anker et al.[9] reported that the MSCs derived from amnion of the placenta are of fetal origin whereas those derived from deciduas are predominantly of maternal origin. Other studies have reported variable results in this regard. For instance, Wulf et al.[112] used whole placenta after removal of the amniotic membranes and found similar amounts of maternal and fetal MSCs in freshly isolated and early passage cells, however later passages (>3rd) contained MSCs of exclusively maternal origin. Similarly, Soncini et al.[104] have shown MSCs of fetal origin in freshly isolated cells from amnion and chorion membranes, but found later passages of MSCs derived from chorion were significantly contaminated with MSCs of maternal origin. In contrast, another study reported that digestion of whole placenta (including amnion, chorion and decidula) produced MSCs of maternal origin.[21] Nevertheless, it is possible to derive MSCs of both fetal and maternal origin from placenta, but fetal MSCs have shown to maintain pluripotency, similar to embryonic stem cells, and carry greater expansion potential than maternal or adult MSCs.[113,114]

While the frequency of MSCs present in bone marrow is relatively low, the frequency of MSCs present in freshly isolated mononuclear cells from digested placenta is yet to be known.[12] On the other hand, current evidence suggests that amniotic fluid contains low number of MSCs which can be effectively expanded in culture.[115] However, when compared with adult bone marrow stem cells both PDSCs and AF-SCs have shown better growth rates and increased differentiation potential.[21,98,99] Furthermore, these cells display immunomodulatory properties and hence they are transplantable between HLA-mismatched individuals without the need for host immunosuppression.[98,105,116] The ready availability of abundant source and extensive expansion potential along with their immunomodulatory properties make PDSCc and AF-SCs as potential sources of MSCs for future therapeutic applications. These cells can be easily mass produced, cryopreserved and shipped to clinics for immediate use; however, as we discussed earlier, the major obstacle for manufacturing clinical grade MSCs have been a need of cGMP grade practices in cell isolation and processing for cryopreservation storage and distribution. For an instance, the PDSCs are normally isolated either using trypsin/flush method or collagen digestion method. But the reagents trypsin and collagenase contain a range of proteins and enzymes that are not considered as cGMP compliant products.[117] As well, before clinical banking of these cells, we must determine that these cells can be grown and cryopreserved in the absence of animal serum. It has been reported that both PDSCs and AF-MSCs require relatively high concentrations of animal serum (~20%) for efficient growth in vitro.[21,117,118] Limited studies have successfully isolated and expanded AF-SCs using commercially available human serum.[118] In a more recent study amnion derived multipotent progenitor cells were cryopreserved and expanded in a serum free culture media before being examined for their immunomodulatory properties.[119] Only one report examined serum free expansion of PDSCs and reported improved proliferation and differentiation when the cells are cultured on a gelatin coated dish in a serum free culture media.[120] However, when the cells were cultured on a non-gelatin coated surface in a serum free media produced a significant loss in proliferation rate.[120] More recently, Huang et al.[107] reported that PDSCs derived from placental decidua basalis can survive under hypoxia and serum deprivation. As we discussed earlier, serum is also added to cryopreservation media for some ill-defined reasons. Our literature survey indicated that, though limited in

number, the published studies mainly focused on developing cryopreservation methods for clinical manufacturing of stem cells derived from amniotic fluid (AF-SCs). In a recently published study which aimed to develop clinical grade manufacturing methods for AF-SCs, it was shown that cryopreservation in the presence of 2.5% human serum AB was efficient in maintaining the post-thaw growth and differentiation potential.[121] In another study, these cells were further shown to be amenable to long-term cryopreservation as they remained viable and potent decades after initial isolation and cryopreservation.[122] So far, the current literature shows PDSCs derived from placental tissues are mainly preserved for research and most of the knowledge in cryopreservation of these cells has been gained from cord blood cryopreservation strategies.[98] More importantly, to date all the cryopreservation methods developed for PDSCs and AF-SCs were commonly employed 10% DMSO as CPA in the presence of either animal or human serum AB. To the best of our knowledge no study has yet investigated the clinical effect of DMSO presence in the cryopreservation media used for PDSCs and AF-ASCs. It remains to be seen how the reduced concentrations of DMSO affects the viability and performance of these cells frozen either in allogeneic/autologous serum or no serum conditions.

Another ideal option to store PDSCs under cGMP conditions is the cryopreservation of large quantities of placental tissue with minimal manipulation immediately after receiving from clinics. The cryopreservation of whole placenta may pose significant technological challenge in terms of freezing and thawing; however minced fragments of placental tissue could provide an alternative choice. Indeed the clinical cryopreservation of human amniotic membranes has been extensively studied for decades in surgery and wound treatment for wound care and decubitus ulcers and in ophthalmology.[98,123] Therefore, this available knowledge on the cryopreservation of amniotic membranes could be used as a starting point for the development of clinical banking strategies for placental tissue sections. Similarly, cryopreservation of uncultivated amniotic fluid with minimal manipulation may be more advantageous for the banking of specimens with no immediate plans for expansion and use.

Freezing, Thawing and Viability Assessment

For all biological systems, the freezing rate is a significant factor in the determination of viability following cryopreserved storage. For the most part, slow freezing protocols seem to be the consensus among most laboratories for cryopreservation of adult stem cells.[12,28,34,36,41] Hunt et al.[124] demonstrated that even with increased concentrations of DMSO, faster cooling rates (10°C/min and 100°C/min) resulted in significant drop-off in viability, and Donaldson et al.[125] even reported a statistically significant difference in viability between cooling at 1 and 5°C/min. Similarly, previous studies with ASCs indicated significant loss in viability when the cooling rate was increased from 1 to 40°C/min.[28] On the other hand, a difference of opinion remains regarding the use of controlled cooling to achieve these rates versus a more simplified uncontrolled method whereby prepared cells are placed into a mechanical freezer for cooling (e.g., 'dump-freezing' method).[94] Other methods of freezing, such as via vitrification using high cooling rates and high concentrations of cryoprotectant, are also being explored. At such high cooling rates freezing does not occur by crystallization or grain growth, but a second-order thermodynamic phase transition leads to an arrest in the translational molecular motions, and the frozen region tends to form amorphous ice, usually referred to as glass. Damage due to intracellular ice formation and high concentration of extracellular solutions solution effects during a typical slow freezing are also avoided. Vitrification is successfully used for human oocytes, embryos and embryonic stem cells[126-128] and for hematopoietic progenitor cells retrieved from human cord blood[129] and for human amnion-derived mesenchymal stem cells.[130] Significant draw back with vitrification is the chemical toxicity and extreme osmotic stresses caused due to the high concentrations of permeable and/or non permeable CPA loading before plunging into liquid nitrogen.[94] Also, vitrification is not a suitable method of cryopreservation for large sample volumes.

For thawing frozen samples, several techniques have already been proposed. The standard method is warming in a water bath at 37°C until all ice crystals disappear. Literature suggests that rapid thawing rates (>100°C/min) that can prevent damaging ice crystals during recrystalization are optimal choice and generally results in the best post-thaw recovery and viability of cells.[131,132] Cryopreservation studies with early passages of ASCs, however, indicated no significant variation in immediate post-thaw viability when the frozen samples are thawed at 10°C/min in controlled rate freezer or in water bath at 37°C (>100°C/min).[28] Additionally, Katayama et al.[133] compared incubation of the frozen peripheral blood stem cells at 4, 20 and 37°C for 20 min and detected significant difference only when the sample is thawed at 4°C. Moreover, guidelines of cGMP favor the thawing performed under defined aseptic conditions. Because of the potential contamination of the water-bath with microorganisms and risk of subsequent transmission to patients, it might be safer to thaw cell suspensions in a dry environment.[132]

Post-thaw viability assessment has shown to be critical in clinical transplantation applications. Choosing the appropriate viability measurement is essential for evaluating the cryopreservation outcome in terms of post-thaw cell quality and quantity. For a routine viability measurement, the trypan blue dye exclusion assay is the most commonly utilized test owing to its easiness and quickness.[134] From the point of view of quality assurance programs meeting the requirement of cGMP, however, this method has the disadvantages that only a small number of cells can be analyzed, that the method is observer dependent, and that it generally overestimates the viable population. Numerous reports have appeared suggesting that fluorescent dyes are more accurate and reliable indicators of cell viability.[134,135] Additionally, recent evidence suggests that the freeze-thawing process induces extensive early apoptosis stress activation pathways which can lead to a time dependant decline in viability and function at culture temperatures.[136-140] Therefore consideration must be given to the adoption of methods that simultaneously detect early apoptotic cells along with necrotic cells for a more accurate assessment of

post-thaw cell viability.[138] For example, de Boer et al.[139] observed a significant reduction in the quantity of viable $CD34^+$ cells mainly due to early but irreversible apoptosis. Recently, Sparrow et al.[140] reported that post-thaw umbilical cord blood samples contained significant portion of apoptotic $CD34^+$ cells and lymphocytes when compared to fresh samples. Since, apoptosis is implicated in the delayed onset of cell death and is accompanied by decline in attachment rate and longtime survival, it is important to perform viability assessment at different time points of post-thaw cultured cells. However, assessments at 24 hours post-thaw allow for a manifestation of most components of the cell stress cascades and encompasses the peak of apoptotic (8–12 hours) and necrotic (4–8 hours) activities.[136-138] To achieve an adequate clinical effect from immediately applied MSCs, it is therefore, very important to evaluate the quality of cryopreserved cells, by including both apoptosis and necrosis and using flow cytometry, before being used in clinics.

Cryopreservation Storage and Shipping

To ensure the utmost quality of cellular products stored for human therapy, the principles of cGMP also applies to the facility design, equipment and devices used for processing, storing and shipping of the cellular products to end user. Physical establishment of stem cell bank should include well organized and properly spaced clean rooms with adequately designed air systems to prevent the spreading of microorganisms from one manufacturing to another. The cGMP regulations also define that within the clean room production unit, only one production process is allowed at a time to avoid cross contamination among different samples.[31] It is also required that stem cell banks maintain stringent environmental microbiological monitoring of the clean rooms where the cell cultures and their products are prepared.[141] Within the establishment, the dedicated space for quality control, change rooms, media and buffer preparation areas, with the implementation of individual hygiene plan is a must.[31]

Stem cells banks should also maintain secure liquid nitrogen storage equipment with the addition of high temperature alarms in order to avoid catastrophic loss of cryopreserved cells. Appropriate labeling of the samples as indicated by the cGMP guidelines is compulsory. Poor labeling and inaccurate documentation of storage inventories can undermine the cryopreservation effort by causing confusion with vial contents and by causing noncompliance with cGMP regulatory standards.[142] In general, storage of preserved material under liquid nitrogen is undesirable for two major reasons. First, the ultra-low temperatures can make the vials to shrink so that the seal may not be tight during storage. This may cause the liquid nitrogen to seep in to vials, which, on rewarming, expand and can subsequently explode as the nitrogen vaporizes inside the vials.[143] Second, the infected product can cross-contaminate other cells stored in the same liquid nitrogen tank.[144-147] These severe cross-contamination issues enforce the stem cells banks to store materials at vapor phase of liquid nitrogen. However, recent evidence suggests that storage in the vapor phase above liquid nitrogen still carries the risk of cross-contamination.[147]

Many stem cells banks use blood bags for storage and for shipping to the clinical site, presumable because they were founded by traditional blood banks with available infrastructure for processing cells into bags, and for the freezing and storing of cells. Compared to bags vials suffer drawbacks in that they are not a closed system, meaning cells are unnecessarily exposed to contaminants during processing and that they are very small and are very prone to temperature fluctuations requiring additional attention during transfer and shipping. While still effective for some indications, recently there has been more focus on the shortcomings of cryopreserved bag storage. A study by Khuu et al.[148] recently investigated a series of catastrophic bag failures first noticed in 2001. While no serious adverse patient effects occurred, extensive fractures led to microbial contamination, increased product preparation time, increased antibiotic use, and increased resource expenditure to replace products. While blood bags and vials are sufficient for small scale processes that generate tens of product doses per lot, commercial scale lot sizes of hundreds to thousands of living cell doses per lot will be required to supply a commercial scale cellular product. Our group recently evaluated the suitability of scalable final container systems for cryopreservation storage of cellular system. These container systems are currently being used to package and deliver pharmaceutical drugs in global markets. Specifically, the Daikyo Crystal Zenith (CZ®) plastic vial system was investigated, which is a subject of drug package development for biologic drugs like peptides, proteins and monoclonal antibodies.[149-151] When DPSCs were used as a model system, our data indicated that CZ vial systems can effectively be utilized to freeze, store and ship cryopreserved DPSCs in a format that enables large scale manufacturing, and fill finish operations and in addition to care givers and patients for ease of handling and delivery.

Shipping cryopreserved samples to clinics ensuring that the quality and utility of the product are maintained during transfer is another pragmatic issue faced by cell banks. The present validated shipping practice is essentially a technological transfer from blood bank industry and organ transplant groups. However, this knowledge may not be sufficient for all cell types. Adult stem cells stored using slow freezing rates are generally shipped on dry ice. Vitrified cells need to be transported at temperatures below its de-vitrification temperature. This precludes the use of dry ice for vitrified samples and necessitates the use of costly dry shippers that maintain the temperature at around -160°C.[127,152] Shipment tracking is essential, knowing where the sample is at any given time. The next major consideration is to provide standardized guidelines to the receiver how the sample should be processed once received. Current cGMP guidelines further advocate that, adequate documentation must accompany a cell sample being shipped to the clinic, including a description of the cells and how they are processed, passage history, its origin and provenance for traceability, actual cell number delivered, cryopreservation method employed, compatibility with the device used for shipping, confirmation of authenticity and freedom from mycoplasma, and biohazard information.[143,153,154] Providing this information to the end user is of paramount importance in the wake of increased incidence of infections in clinical transplantations due to contaminated cell populations.

Conclusions

While adult stem cells are increasingly used in clinical trials, the manufacturing of clinical grade MSCs poses substantial challenge to scientists all over the world. Although cGMP practices generate high quality cellular products, the risk of product contamination will not necessarily be eliminated. This may in part due to the fact that implementation of stringent cGMP regulations is indeed impractical in scale-up manufacturing processes that are generally used in stem cell banks. This is because of both impossibility of absolute elimination of animal based products without the loss of valuable biological activity and the absence of widely accepted and fully established serum free alternatives for processing and storage. Therefore, new research is required to focus not only on eliminating xenogenic components during processing of cellular products but also on viable techniques to remove these animal components in the final products before they are being used in clinical therapy. Additionally, elimination of cryoprotectants and animal products during freezing and thawing, the quality of liquid nitrogen, materials and methods to store and deliver the components to end user are also major constituents in a GMP compatible manufacturing plant. More importantly, there is clearly a lack of clarity in FDA regulations on the processing and storage of cells for clinical applications, and there is a critical need for consensus among manufacturers of cell products with respect to these issues.

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